

# Vitamin D as Supplemental Therapy for *Pneumocystis* Pneumonia

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The combination of all-*trans* retinoic acid (ATRA) and primaquine (PMQ) has been shown to be effective for therapy of *Pneumocystis* pneumonia (PCP). Since a high concentration of ATRA has significant adverse effects, the possibility that vitamin D can be used to replace ATRA for PCP therapy was investigated. C57BL/6 mice were immunosuppressed by depleting CD4<sup>+</sup> cells and infected with *Pneumocystis murina* 1 week after initiation of immunosuppression. Three weeks after infection, the mice were treated orally for 3 weeks with vitamin D<sub>3</sub> (VitD3) alone, PMQ alone, a combination of VitD3 and PMQ (VitD3-PMQ), or a combination of trimethoprim and sulfamethoxazole (TMP-SMX). Results showed that VitD3 (300 IU/kg/day) had a synergistic effect with PMQ (5 mg/kg/day) for therapy of PCP. Flow cytometric studies showed that this VitD3-PMQ combination recovered the CD11b<sup>low</sup> CD11c<sup>high</sup> alveolar macrophage population in mice with PCP as effectively as TMP-SMX. The VitD3-PMQ combination also reduced the massive infiltration of inflammatory cells into the lungs and the severity of lung damage. VitD3 was also shown to reduce the dose of TMP-SMX required for effective treatment of PCP. Taken together, results of this study suggest that a VitD3-PMQ combination can be used as an alternative therapy for PCP.

*Pneumocystis jirovecii* is the causative organism of *Pneumocystis* pneumonia (PCP), which is a common opportunistic disease in patients with AIDS. According to the 2014 CDC statistics, the incidence of PCP in the United States is 9% among hospitalized AIDS patients and 1% among solid organ transplant recipients (1). The mortality rate of PCP ranges from 5% to 40% in patients with treatment and approaches 100% in those without treatment (1). *Pneumocystis* organisms are also found in other mammalian species, and those found in mice are called *P. murina*. *P. carinii* refers to the major form of *Pneumocystis* found in rats. Although *Pneumocystis* organisms are classified as fungi, they are not susceptible to most antifungal drugs. Currently, the most effective regimen for PCP therapy is the combination of trimethoprim and sulfamethoxazole (TMP-SMX). However, approximately 10% of people are allergic to sulfa drugs (2), and many AIDS patients fail therapy with TMP-SMX (3). In addition, TMP-SMX may cause adverse effects, such as rash, fever, neutropenia, thrombocytopenia, or transaminase elevation (3).

In PCP, alveolar macrophages (AMs) are defective in phagocytosis and reduced in number (4). We have found that the expression of the PU.1 gene in AMs is decreased during PCP (5). This PU.1 downregulation may be a cause of AM dysfunction because PU.1 regulates the expression of many macrophage receptors (5–9). Since PU.1 also regulates the differentiation of monocytes into AMs (10), its decrease in expression would adversely affect this process, resulting in a decreased number of AMs during PCP. We have also found that myeloid-derived suppressor cells (MDSCs) accumulate in the lungs of mice and rats with PCP (11). The number of these cells increases as *Pneumocystis* infection becomes more severe and decreases with successful TMP-SMX treatment, suggesting that *Pneumocystis* infection causes MDSC accumulation. Surprisingly, treatment of *Pneumocystis*-infected mice and rats with all-*trans* retinoic acid (ATRA), a metabolite of vitamin A, for 5 weeks was found to clear the infection, with the disappearance of MDSCs in their lungs (11). These findings suggest that MDSCs are incompletely differentiated monocytes and that ATRA treatment stimulates them to differentiate into AMs, allowing the host to defend the infection.

We have also found that ATRA (5 mg/kg of body weight/day)

in combination with primaquine (PMQ) (2 mg/kg/day) is as effective as TMP-SMX for PCP therapy and clears the infection in 2 weeks (12). Because ATRA has significant adverse effects (13), we tested whether ATRA can be replaced with vitamin D, as it has been shown to stimulate MDSCs to differentiate into antigen-presenting cells (14, 15). Vitamin D is synthesized in the skin by first converting 7-dehydrocholesterol (provitamin D<sub>3</sub>) to precholecalciferol (previtamin D<sub>3</sub>) upon exposure to sunlight. At body temperature, precholecalciferol is converted to cholecalciferol, which is vitamin D<sub>3</sub> (VitD3). Another source of vitamin D is food, such as eggs and fatty fish. Vitamin D is measured in international units (IU) (25 ng/IU) in nutritional supplements and in nanograms per milliliter or nanomoles per liter in serum or plasma (1 nmol/liter = 0.4006 ng/ml). Once produced in the skin or absorbed from the gut, vitamin D is carried by vitamin D-binding protein and transported to the liver, where it is metabolized to 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>] (also called calcidiol) by 25-hydroxylase (CYP2R1). 25-Hydroxyvitamin D<sub>3</sub> is then converted in the kidney by 25-OH-D<sub>3</sub>-1 $\alpha$ -hydroxylase (CYP27B1) to 1,25-dihydroxyvitamin D<sub>3</sub> [1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>] (also called calcitriol), which is the active form of vitamin D. Results of this study showed that vitamin D<sub>3</sub> (VitD3) supplementation decreased the severity of *Pneumocystis* infection. Furthermore, VitD3 was found to have a synergistic effect with PMQ for PCP therapy.

## MATERIALS AND METHODS

**Mouse model of PCP.** C57BL/6 mice were obtained from Harlan (Indianapolis, IN). All mice used in this study were female, 18 to 20 g in weight. The study was approved by the Indiana University Animal Care and Use

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Committee and carried out under the supervision of veterinarians. For immunosuppression, each mouse was injected intraperitoneally with 0.3 mg anti-L3T4 monoclonal antibody (MAb) (clone GK1.5; Harlan, Indianapolis, IN) once a week to deplete CD4<sup>+</sup> cells for the entire period of the study. This method of immunosuppression has been shown to be effective (16) and has been used in many PCP studies (5, 11, 12, 17). One week after the initial injection of the antibody, each immunosuppressed mouse was transtracheally inoculated with  $2 \times 10^6$  *P. murina* organisms in 30  $\mu$ l phosphate-buffered saline (PBS). The *Pneumocystis* organisms used as inoculum were obtained from heavily infected lungs and isolated as previously described (4). Mice were given drinking water containing tetracycline (0.74 g/liter) to prevent bacterial infections as previously described (5, 11, 12).

**Drug treatment.** The following regimens were used: TMP-SMX (TMP, 50 mg/kg/day; SMX, 250 mg/kg/day), ATRA-PMQ (ATRA, 5 mg/kg/day in 8% dimethyl sulfoxide [DMSO]; PMQ, 2 mg/kg/day in water), VitD3 (300 IU/kg/day in 8% DMSO), PMQ (5 mg/kg/day), VitD3-PMQ (VitD3, 300 IU/kg/day; PMQ, 5 mg/kg/day). VitD3 was used at 300 IU/kg/day, as our preliminary studies showed no difference in efficacy between this dose and 3,000 IU/kg/day (data not shown). All drugs were given orally by gavage. Five mice in each group were sacrificed for investigation of drug effects at a specific time point. TMP-SMX (Septra), in liquid form, was purchased from Hi-Tech Pharmacal (Amityville, NY). ATRA, VitD3 (cholecalciferol), and PMQ were obtained from Sigma-Aldrich (St. Louis, MO).

**Evaluation of *Pneumocystis* infection.** Mice were anesthetized by intramuscular injection of ketamine cocktail (ketamine hydrochloride, 90 mg/kg; acepromazine, 1.5 mg/kg; xylazine, 2.5 mg/kg) and then sacrificed by cardiac exsanguination. The lungs of the mice in each group were removed, fixed with buffered 10% formalin overnight, and then embedded in paraffin as described previously (11). Five-micrometer histologic sections were stained with hematoxylin and eosin (H&E) to evaluate lung inflammation and with Grocott's methenamine silver (GMS) to assess organism load by microscopy.

**BAL.** Lungs were lavaged with sterile saline (1 ml at a time) through an intratracheal catheter until a total of 10 ml of lavage fluid was recovered as described previously (18). The bronchoalveolar lavage fluid (BALF) was centrifuged at  $300 \times g$  for 10 min to pellet BAL cells. The pelleted cells were washed twice with PBS and then resuspended in 1 ml PBS.

**Flow cytometry of BAL cells.** BAL cells were incubated with 5% bovine serum albumin for 1 h and then reacted with anti-mouse phycoerythrin (PE)-labeled anti-CD11b (catalog no. 101207; BioLegend) and Alexa Fluor 647-labeled anti-CD11c (catalog no. 117314; BioLegend) antibodies on ice for 1 h. Control cells were stained with PE- or fluorescein isothiocyanate (FITC)-labeled isotype IgG. The antibody-stained cells were washed with 4 ml PBS and then examined with a BD FACSCalibur flow cytometer (BD Biosciences) as described previously (11). FlowJo software (Tree Star, Ashland, OR) was used to analyze the flow cytometry data.

**Determination of albumin and LDH levels in BALF.** The first 1 ml of BALF was used for determination of albumin and lactate dehydrogenase (LDH) levels. The albumin concentration was measured using a QuantiChrom BCG albumin assay kit (BioAssay Systems, Hayward, CA), and LDH levels were determined using an LDH cytotoxicity assay kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturers' instructions.

**Measurement of vitamin D<sub>3</sub> and calcium levels in mouse serum.** Mouse blood was collected by cardiac puncture and then placed in a 37°C incubator for 30 min. After centrifugation of the clotted blood for 15 min at  $1,500 \times g$  at 4°C, the clarified serum was transferred to a new tube. Serum VitD3 levels were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (19). Control 25(OH)D3 and deuterated vitamin D [ $d_6$ -25(OH)D] were purchased from Cerilliant (Round Rock, TX). LC/MS-grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). The internal standard consisted of a stock solution of 5  $\mu$ g/ml of  $d_6$ -

25(OH)D in methanol diluted with acetonitrile to a final concentration of 75 ng/ml.

A total of seven 25(OH)D3 calibration standards with final concentrations of 0 to 150 ng/ml in 50% methanol were prepared. To measure VitD3 levels in serum, 200  $\mu$ l of precipitating reagent (acetonitrile) with internal standard [ $d_6$ -25(OH)D] was added to 100  $\mu$ l of mouse serum samples, control samples, and calibrator samples. After vortexing for 30 s, samples were centrifuged at  $4,000 \times g$  for 10 min to pellet the insoluble substances. The supernatant of each sample was transferred to an autosampler vial and sealed.

Solvent delivery and sample introduction were performed using a ThermoScientific ARIA/Transcend TLX2 LC system with pumps and ThermoScientific (San Jose, CA) high-performance liquid chromatography (HPLC) columns. After injection (50  $\mu$ l) at room temperature, additional online sample preparation was performed using a TurboFlow C<sub>18</sub>-P XL column (50 by 0.5 mm). Chromatographic separation of VitD3 from other substances was performed on an Accucore C<sub>18</sub> column (50 by 3 mm) at room temperature. Detection of VitD3 was performed on a TSQ Vantage triple-quadrupole mass spectrometer (ThermoScientific, San Jose, CA). Quantitation of VitD3 was performed using TraceFinder 3.0 version 3.0.742.16 software (ThermoScientific) by a comparison with the standard curve derived from the seven 25(OH)D3 calibration standards.

The total calcium measurement was based on the observation that a purple Ca-arsenazo III complex is formed when calcium ion is reacted with arsenazo III (2,2'-[1,8-dihydroxy-3,6-disulfonaphthylene-2,7-bisazo]-bisbenzenearsonic acid) (20). For each measurement, 2.5  $\mu$ l of serum was mixed with 21  $\mu$ l of arsenazo III and diluted with 150  $\mu$ l of water (OSR65117; Beckman-Coulter) on a Beckman-Coulter (Brea, CA) AU680 analyzer. The resulting Ca-arsenazo III complex was quantified spectrophotometrically at 660 nm.

**Statistic analysis.** An unpaired two-tail Student *t* test was performed to compare the difference between treatment and control (untreated) groups or the efficacy of VitD3-PMQ to that of TMP-SMX, PMQ alone, or VitD3 alone in PCP therapy. The data presented are means  $\pm$  standard deviations (SD). A *P* value of  $<0.05$  is considered significant.

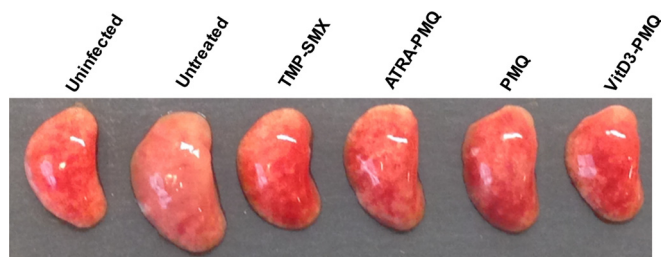
## RESULTS

### Effective treatment of PCP by a combination of VitD3-PMQ.

Experiments were first performed to investigate whether VitD3 can replace ATRA and whether it has any synergistic effect with PMQ for PCP therapy. Mice were infected with *Pneumocystis* 1 week after initiation of immunosuppression. The infected mice (referred to here as PCP mice) were treated with a combination of VitD3 (300 IU/kg/day) and PMQ (2 mg/kg/day), starting at 3 weeks after infection. Control PCP mice were treated with ATRA-PMQ (ATRA, 5 mg/kg/day; PMQ, 2 mg/kg/day) or TMP-SMX as previously described (12). Results showed that this combination of VitD3 and PMQ was not as effective as the ATRA-PMQ combination and did not completely clear *Pneumocystis* infection after 3 weeks of treatment. Therefore, the dose of PMQ was increased to 5 mg/kg/day and used in combination with 300 IU/kg/day of VitD3. To evaluate the efficacy of this new VitD3-PMQ regimen, the gross lung morphology was first examined. Untreated PCP mice were found to have greatly enlarged lungs. No significant enlargement of lungs was observed in PCP mice treated with TMP-SMX, ATRA-PMQ, PMQ alone (5 mg/kg/day), or VitD3-PMQ (Fig. 1). These results suggest that the combination of VitD3 at 300 IU/kg/day and PMQ at 5 mg/kg/day is effective for treatment of PCP. This VitD3 and PMQ combination (referred to here as VitD3-PMQ) was used for all subsequent studies.

**Reduction in lung inflammation by VitD3-PMQ treatment.** To confirm the therapeutic effect of VitD3-PMQ, lung sections of uninfected, untreated PCP mice and PCP mice treated with TMP-

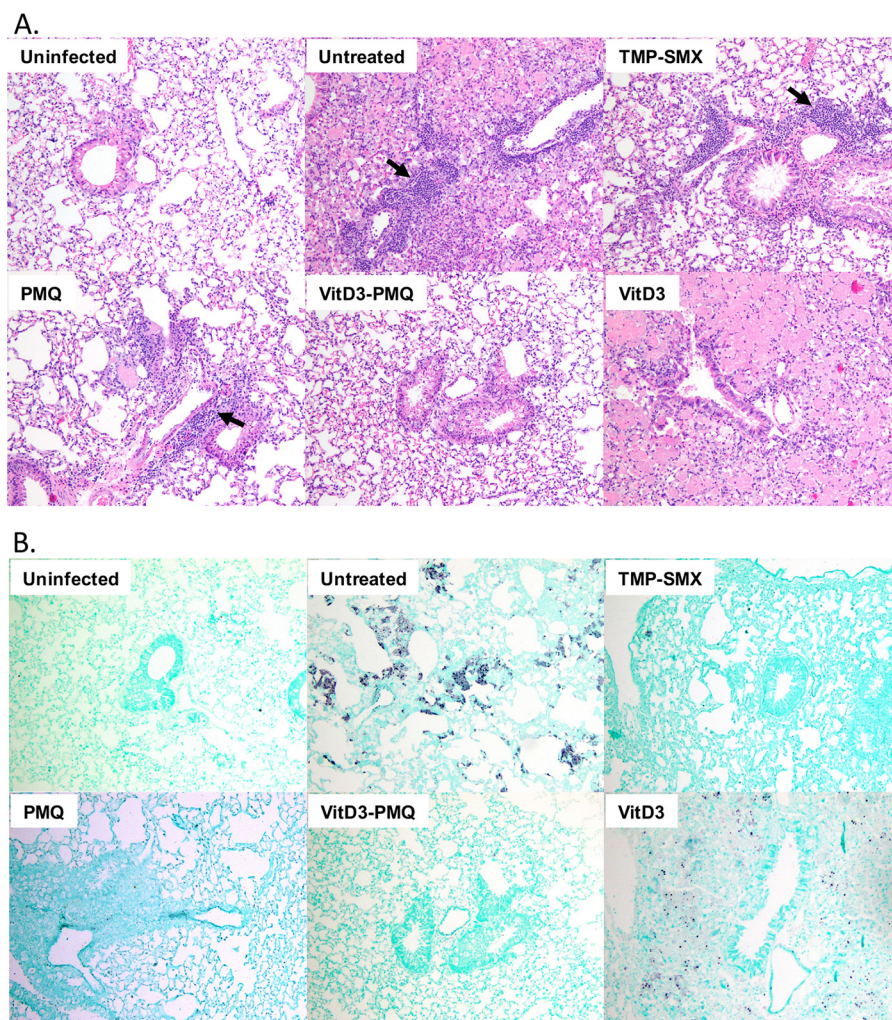




**FIG 1** Gross lung morphology. Immunosuppressed mice were infected with *Pneumocystis* for 3 weeks and then treated with the following regimens for 3 weeks: TMP-SMX, ATRA-PMQ (5 mg ATRA/kg/day and 2 mg PMQ/kg/day), PMQ (5 mg/kg/day), or VitD3-PMQ (300 IU VitD3/kg/day and 5 mg PMQ/kg/day). Representative images of lungs from various groups of mice are shown. Controls are lungs from uninfected mice and untreated PCP mice.

SMX, PMQ alone, VitD3-PMQ, or VitD3 alone were examined. Hematoxylin and eosin (H&E) staining of the lung sections showed the typical severe inflammation and foamy exudates in the lungs of untreated PCP mice after 6 weeks of infection. TMP-SMX

or PMQ treatment of PCP mice for 3 weeks (starting at 3 weeks after infection) greatly reduced the severity of inflammation, but mild inflammation was still present (Fig. 2A). Treatment of PCP mice with VitD3-PMQ for 3 weeks reduced the severity of inflammation much more than that with PMQ alone and almost completely eliminated inflammatory cells. No or minimal foamy exudates were seen in PCP mice treated with TMP-SMX, PMQ alone, or VitD3-PMQ. In those treated with VitD3 alone for 3 weeks, no significant inflammatory infiltrate was observed, despite the presence of enormous amounts of foamy exudates in alveoli (Fig. 2A). Methenamine silver staining revealed numerous *Pneumocystis* organisms in the lung sections of untreated PCP mice but no organisms in those of TMP-SMX-, PMQ-, or VitD3-PMQ-treated PCP mice. The organism load in the lung sections of PCP mice treated with VitD3 alone was much lower than that of untreated PCP mice (Fig. 2B). In addition, mice treated with VitD3-PMQ were more physically active and gained more weight each week than did the mice in any other group. The mean weight gain per week for uninfected mice was 0.56 g, and that for untreated PCP mice was 0.29 g ( $P = 0.026$ , compared to untreated group). TMP-SMX-



**FIG 2** Lung histopathology. The following groups of mice were investigated: uninfected, untreated PCP mice, and PCP mice treated with TMP-SMX, PMQ, or VitD3-PMQ for 3 weeks. (A) Representative H&E-stained lung sections. Arrows indicate infiltrated inflammatory cells. (B) Lung sections stained with Grocott's methenamine silver. *Pneumocystis* cysts are seen as black dots or clusters.

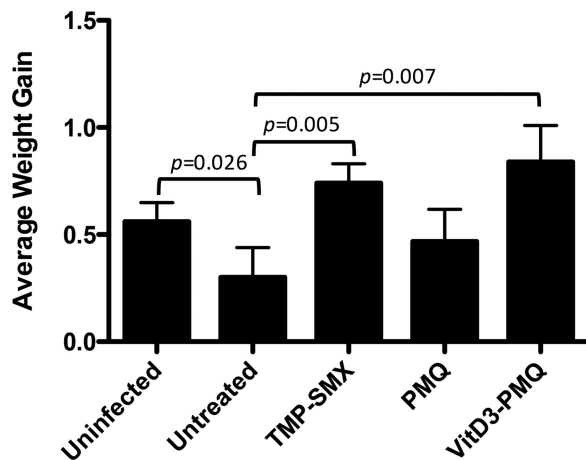


FIG 3 Body weight variation. The body weight of each mouse in the animal groups described in the legends to Fig. 1 and 2 was determined once a week. The average weight gain per week of each group of mice is plotted. Data are presented as means  $\pm$  SD of results for 5 mice for each group.

treated PCP mice gained an average of 0.74 g ( $P = 0.005$ , compared to untreated group), and those treated with PMQ alone gained 0.47 g per week. Surprisingly, VitD3-PMQ-treated PCP mice gained an average of 0.84 g per week ( $P = 0.007$ , compared to untreated group), 0.28 g higher than that in uninfected mice. (Fig. 3).

**Recovery of AMs by VitD3-PMQ treatment.** To determine the effect of various treatments on the number of AMs, flow cytometric examinations of BAL cells were performed using PE-labeled anti-CD11b and Alexa Fluor 647-labeled anti-CD11c antibodies. Results showed that the CD11b<sup>low</sup> CD11c<sup>high</sup> AM populations in uninfected immunosuppressed mice were 91.2%, 82.5%, and 86.2% of total BAL cells at 5, 6, and 7 weeks, respectively, after initiation of immunosuppression. In untreated PCP mice, this population of cells was decreased to 32% at 4 weeks, 11.3% at 5 weeks, and 1.95% at 6 weeks after infection. In TMP-SMX-treated mice, the CD11b<sup>low</sup> CD11c<sup>high</sup> AM population was 66.9% after 1 week of treatment and increased to 73.2% at 2 weeks and 80.5% at 3 weeks after treatment. This population of cells was 42.2% after 1 week and increased to 57.5% after 2 weeks and 74.8% after 3 weeks of PMQ treatment. In VitD3-PMQ-treated PCP mice, the CD11b<sup>low</sup> CD11c<sup>high</sup> AM population was 71% at 1 week, 87.7% at 2 weeks, and 89.2% at 3 weeks after treatment. The most striking features in mice with PCP were the gradual loss of the CD11b<sup>low</sup> CD11c<sup>high</sup> AM population and the increase of the CD11b<sup>high</sup> CD11c<sup>low</sup> MDSC population. *Pneumocystis*-infected mice treated with TMP-SMX, PMQ, or VitD3-PMQ for 3 weeks recovered the CD11b<sup>low</sup> CD11c<sup>high</sup> AM population to levels close to those of uninfected mice (Fig. 4A and B).

**Reductions in albumin and LDH levels by VitD3-PMQ treatment.** To evaluate lung injury, the levels of albumin and LDH in BALF of the following animal groups were determined: uninfected mice, untreated PCP mice, and PCP mice treated with TMP-SMX, PMQ, or VitD3-PMQ for 3 weeks. As shown in Fig. 5A, albumin levels in the first BALF collected from uninfected mice were 6.39 mg/dl but were greatly elevated (26.22 mg/dl,  $P = 0.007$ ) in those of untreated PCP mice. Treatment of PCP mice with PMQ alone (7.53 mg/dl) or VitD3-PMQ (8.35 mg/dl) re-

duced BALF albumin to levels similar to those of mice treated with TMP-SMX (9.3 mg/dl). The levels of LDH in the BALF of untreated PCP mice were approximately five times higher than those of uninfected mice (852.63  $\mu$ U versus 160.8  $\mu$ U,  $P = 0.001$ ). Treatment of PCP mice with TMP-SMX reduced their BALF LDH levels to 281.73  $\mu$ U ( $P = 0.003$ , compared to untreated group), close to those of uninfected mice. Treatment of PCP mice with PMQ (323.33  $\mu$ U,  $P = 0.004$ , compared to untreated group) or VitD3-PMQ (276.27  $\mu$ U,  $P = 0.003$ , compared to untreated group) reduced LDH levels by approximately 70% (Fig. 5B).

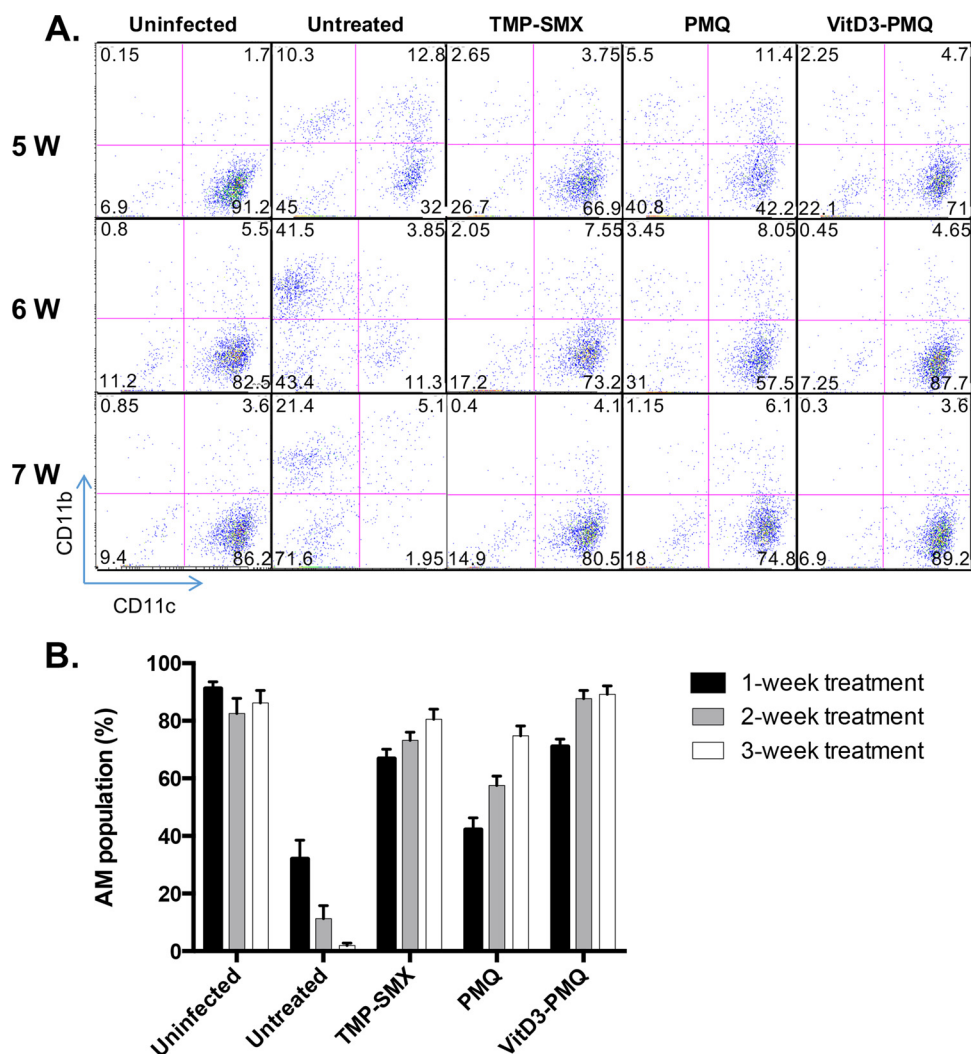
**No increase in serum calcium levels after VitD3-PMQ treatment.** To ensure that oral administration of VitD3 was effective, serum vitamin D [25(OH)D3] levels of the following groups of mice were measured: uninfected mice, untreated PCP mice, and PCP mice treated with TMP-SMX, PMQ, or VitD3-PMQ. At the time of vitamin D measurement, untreated PCP mice were infected with *Pneumocystis* for 6 weeks, and *Pneumocystis*-infected mice were treated with TMP-SMX, PMQ alone, or VitD3-PMQ for 3 weeks, starting at 3 weeks after *Pneumocystis* infection. The mean serum 25(OH)D3 levels of the various groups were as follows: uninfected mice, 31.93 ng/ml; untreated PCP mice, 34.75 ng/ml; PCP mice treated with TMP-SMX, 29.6 ng/ml; PCP mice treated with PMQ, 32.67 ng/ml; and PCP mice treated with VitD3-PMQ, 113.8 ng/ml (Fig. 6A). To determine whether vitamin D treatment caused hypercalcemia, calcium concentrations in the sera of these groups of mice were determined. Results showed that serum calcium levels in uninfected mice, untreated PCP mice, and PCP mice treated with TMP-SMX, PMQ, or VitD3-PMQ were all about the same (9 mg/dl) (Fig. 6B). Therefore, VitD3 treatment did not raise serum calcium levels, even though serum 25(OH)D3 levels of treated mice were approximately 4-fold that of untreated mice.

**Reduction in TMP-SMX dose by vitamin D.** To determine whether VitD3 can reduce the dose of TMP-SMX required for PCP therapy, combinations of VitD3 with full (50 mg TMP plus 250 mg SMX per kg/day) and one-third doses of TMP-SMX were tested on mice that had been infected with *Pneumocystis* for 3 weeks. Results showed that VitD3 at 300 IU/kg/day reduced the dose of TMP-SMX required for effective therapy of PCP. As shown in Fig. 7A, numerous *Pneumocystis* cysts are present in methenamine silver-stained lung sections from PCP mice treated with one-third dose of TMP-SMX alone at 1, 2, and 3 weeks after treatment, but few are seen in sections from those treated with the combination of VitD3 and one-third dose of TMP-SMX even just 1 week of treatment. The combination of VitD3 with one-third dose of TMP-SMX was also as effective as a full dose of TMP-SMX in the control of lung inflammation, as the number of infiltrated inflammatory cells was greatly decreased after 2 or 3 weeks of either treatment (Fig. 7B). These results suggest that VitD3 also has a synergistic effect with TMP-SMX.

## DISCUSSION

In this study, we found that VitD3 has a synergistic effect with PMQ for the treatment of PCP. This property is similar to that of ATRA, which we have shown to be effective for PCP therapy when it was used in combination with PMQ (12). However, a higher dose of PMQ (5 instead of 2 mg/kg/day) was required when it was used in combination with VitD3 (300 IU/kg/day) for therapy of PCP. The major therapeutic effect of VitD3 was the reduction of the infiltration of inflammatory cells into the lungs, as PCP mice





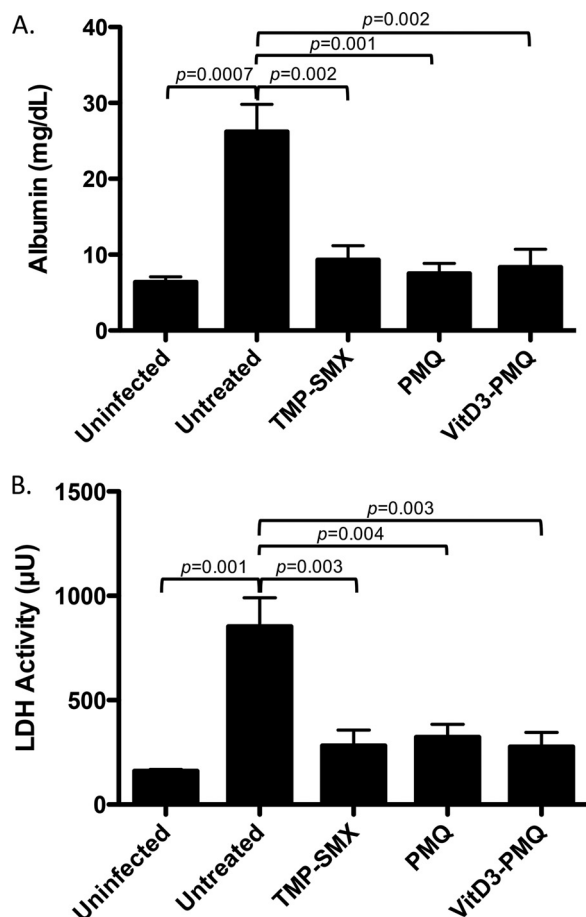
**FIG 4** Flow cytometry of BAL cells. Immunosuppressed mice were infected with *Pneumocystis* and then treated with TMP-SMX, PMQ, or VitD3-PMQ. BAL cells from PCP mice immunosuppressed for 5, 6, and 7 weeks were examined. For treated mice, these three time points correspond to 1, 2, and 3 weeks of treatment. BAL cells from various groups of mice were stained with PE-labeled anti-CD11b and Alexa Fluor 647-labeled anti-CD11c antibodies and then examined. (A) Flow cytometry data. The percentage of cells in each quadrant is indicated. (B) Histogram of CD11b<sup>low</sup> CD11c<sup>high</sup> AM populations of various groups of mice.

treated with VitD3-PMQ for 3 weeks had significantly fewer inflammatory cells in the lungs than those treated with TMP-SMX for the same length of time (Fig. 2A). VitD3 treatment also reduced the organism burden, even in PCP mice treated with VitD3 alone (Fig. 2B).

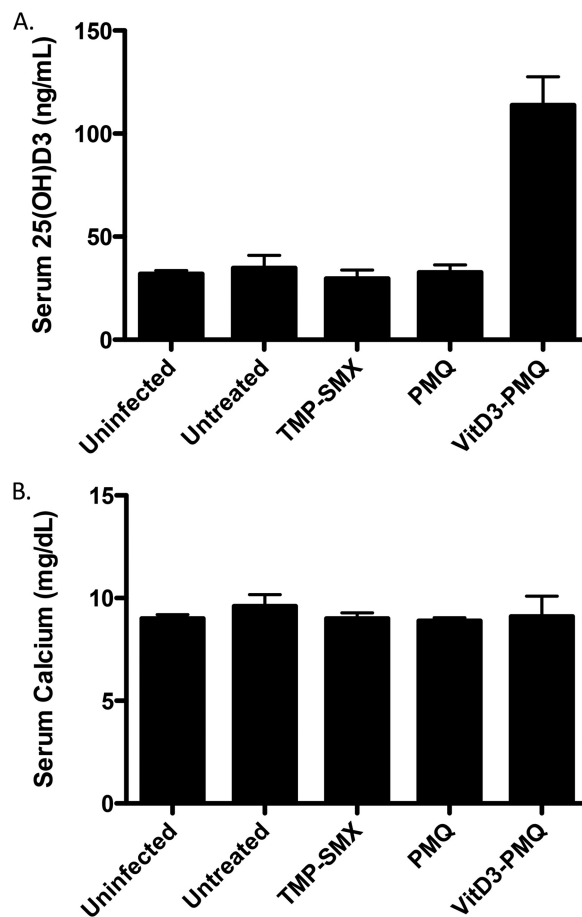
As shown in Fig. 4A, the CD11c<sup>high</sup> CD11b<sup>low</sup> AM population was greatly decreased in untreated PCP mice. Similar to PCP mice treated with TMP-SMX, PCP mice treated with VitD3-PMQ and PMQ alone recovered the CD11c<sup>high</sup> CD11b<sup>low</sup> AM population (Fig. 4A and B). Although TMP-SMX was effective for PCP therapy, it rendered treated animals less active than uninfected mice. In contrast, mice treated with VitD3-PMQ behaved like uninfected ones, with greater weight gain (Fig. 3). This observation suggests that VitD3-PMQ is less toxic than TMP-SMX. Since sulfamethoxazole is a component of TMP-SMX and many people are allergic to it (2), the use of VitD3-PMQ for therapy of PCP would avoid this adverse reaction. In addition, VitD3 supplementation was found to reduce the dose of TMP-SMX required for effective

treatment of PCP by 67% (Fig. 7A and B). As TMP-SMX is currently the first-line drug for PCP, patients are usually treated with it as soon as PCP is suspected. A reduced dose of TMP-SMX would decrease the risk or severity of sulfa drug hypersensitivity. The cost of the VitD3-PMQ combination is about the same as that of TMP-SMX.

In addition to suppressing inflammatory responses (21), vitamin D is known to enhance the production of antibacterial proteins such as cathelicidin and  $\beta$ -defensin, the formation of autophagosomes, and the killing of microorganisms by macrophages (22). Another possible mechanism of action of vitamin D is regulation of the expression of its target genes. Vitamin D works through vitamin D receptor (VDR), which is a nuclear receptor and is present in many types of cells (23). Vitamin D has both genomic and nongenomic effects (24). For the genomic effect, vitamin D binds to nuclear VDR, enabling it to form heterodimers with the retinoid-X receptor (RXR). The vitamin D-VDR-RXR complex acts as a transcription factor and binds to the



**FIG 5** Albumin and LDH levels in BALF. The first milliliter of BALF collected was used for the determination of albumin and LDH levels. (A) Albumin levels in BALF of uninfected mice, untreated PCP mice, and PCP mice treated with TMP-SMX, PMQ, or VitD3-PMQ for 3 weeks. (B) LDH levels in BALF of the same animal groups as described for panel A.



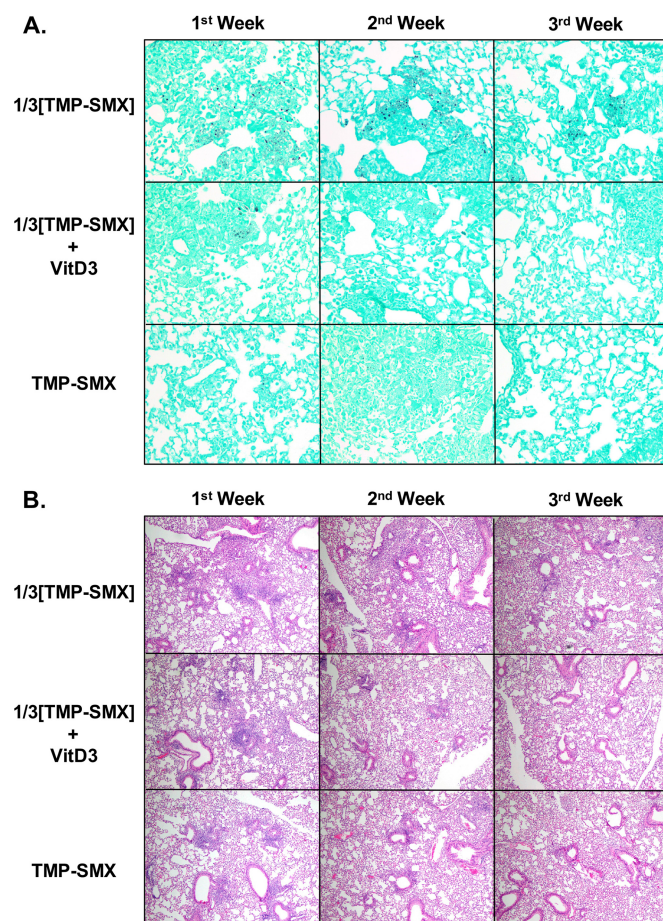
**FIG 6** Serum 25(OH)D3 and calcium levels. Immunosuppressed mice were infected with *Pneumocystis* for 4 weeks and then treated with various regimens. (A) Serum 25(OH)D3 levels in uninfected mice, untreated PCP mice, and PCP mice treated with TMP-SMX, PMQ, or VitD3-PMQ for 3 weeks. (B) Total serum calcium levels of the same animal groups as described for panel A.

vitamin D response element (VDRE), which is present in the promoter region of more than 900 vitamin D-VDR target genes (25). These genes play major roles in various activities, such as calcium and phosphate homeostasis, apoptosis, and cell differentiation (25). For the nongenomic effect, vitamin D binds to VDR located in caveolae in the plasma membrane and exerts its effects through second messengers, such as phospholipase C (PKC), protein kinase C, G protein-coupled receptors, or phosphatidylinositol-3-kinase (PI3K) (26). Pathways that may be activated by the nongenomic effects of the vitamin D-VDR complex include those that are dependent on mitogen-activated protein kinase (27), cyclic AMP (cAMP) (28), phospholipase A2 (29), phospholipase C (30), PI3K (31), or protein kinase C (32). It remains to be investigated whether vitamin D acts through these mechanisms in the supplemental PCP therapy.

PMQ is an 8-aminoquinoline. It is normally used in combination with chloroquine to treat malaria caused by *Plasmodium falciparum* (33). A potential adverse effect of PMQ is an increase in the levels of methemoglobin, in which the ferric ion is replaced by a ferrous ion. Normally, the methemoglobin level is less than 1%. A regimen of 15 mg/day PMQ for 14 days has been shown to raise the methemoglobin level to 5.8% (33). However, a level of less

than 20% methemoglobin is usually asymptomatic. PMQ has been used at 30 mg daily for a year (34) and 60 mg/kg/day for 7 days without any adverse effects (35). The 5-mg/kg/day dose we have used in this study in mice is equivalent to 0.3 mg/kg/day (or 18 mg for 60 kg of body weight) in humans, because the effective dose for mice is approximately 10-fold higher than that for humans (36). PMQ has been used at 15 to 30 mg per day in combination with clindamycin (1,800 mg/day) as the second-line drug for treatment of PCP (37). Another side effect of PMQ is acute hemolysis in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency (33). Therefore, the VitD3-PMQ combination is not recommended for PCP patients with G6PD deficiency.

The replacement of ATRA-PMQ with VitD3-PMQ is a significant improvement, as ATRA may cause fever, white blood cell elevation, respiratory distress, interstitial pulmonary infiltrates, pleural and pericardial effusion, dyspnea, hypotension, or renal failure (13). It has been shown that a daily uptake of 10,000 IU of VitD3 is safe in humans (38, 39). The only potential side effect of high levels of vitamin D is hypercalcemia ( $>12$  mg/dl) (40), which may increase the risk of developing kidney stones. However, this side effect has been observed only in individuals taking more than



**FIG 7** Reduction of required TMP-SMX dose by VitD3. Methenamine silver-stained (A) and H&E-stained (B) lung sections of PCP mice treated with a full dose of TMP-SMX, one-third dose of TMP-SMX, or one-third dose of TMP-SMX plus VitD3. Black dots in the methenamine silver-stained sections are *Pneumocystis* cysts.

50,000 IU/day with a serum 25(OH)D concentration greater than 700 nmol/liter (280 ng/ml) (39). Therefore, a serum 25(OH)D level of <600 nmol/liter (240 ng/ml) is considered safe (39). Without vitamin D supplementation, serum 25(OH)D levels are in the range of 20 to 50 ng/ml (50 to 125 nmol/liter) in humans (41–46) and 34 to 50 ng/ml (85 to 125 nmol/liter) in C57BL/6 mice (47). In this study, we found that the mean serum 25(OH)D level was 31.93 ng/ml in uninfected mice and 34.75 ng/ml in untreated PCP mice. Treatment of PCP mice with VitD3-PMQ raised serum 25(OH)D levels to 113.8 ng/ml (Fig. 6A). Although this concentration is approximately 4 times higher than that of untreated mice, no increase in serum calcium levels was observed (Fig. 6B). There have been attempts to develop  $1\alpha,25(\text{OH})_2\text{D}_3$  analogues without the hypercalcemic side effect. One such example is the development of MART-10 [19-nor- $2\alpha$ -(3-hydroxypropyl)- $1\alpha,25$ -dihydroxyvitamin  $\text{D}_3$ ], which does not raise serum calcium levels (48, 49) and has been shown to be 100-fold more potent than  $1\alpha,25(\text{OH})_2\text{D}_3$  in inhibiting the growth of several types of cancer cells *in vitro* (49–51).

The mode of action of VitD3 supplementation in PCP therapy is currently unknown and is being investigated. We speculate that similar to ATRA, VitD3 prevents the formation of MDSCs and

promotes the differentiation of AMs. It is also possible that VitD3 enhances AMs to produce antimicrobial peptides such as cathelicidin. These potential actions can increase the self-defense ability of *Pneumocystis*-infected hosts. PMQ has been shown to suppress the proliferation of *Pneumocystis* organisms (52). The combination of these two different mechanisms would make the VitD3-PMQ combination ideal for PCP therapy. It is possible that vitamin D also has synergistic effects with other drugs, such as atovaquone and dapsone. These drugs are not used as often as TMP-SMX because they are less effective (53, 54). However, they are less toxic than TMP-SMX (54–56). If VitD3 supplementation can also enhance the efficacy of these drugs, we will have more alternative treatments for PCP patients who cannot tolerate TMP-SMX.

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